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Characterization of Enterohemorrhagic *Escherichia coli* Strains Based on Acid Resistance Phenotypes†

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Acid resistance is perceived to be an important property of enterohemorrhagic *Escherichia coli* strains, enabling the organisms to survive passage through the acidic environment of the stomach so that they may colonize the mammalian gastrointestinal tract and cause disease. Accordingly, the organism has developed at least three genetically and physiologically distinct acid resistance systems which provide different levels of protection. The glutamate-dependent acid resistance (GDAR) system utilizes extracellular glutamate to protect cells during extreme acid challenges and is believed to provide the highest protection from stomach acidity. In this study, the GDAR system of 82 pathogenic *E. coli* isolates from 34 countries and 23 states within the United States was examined. Twenty-nine isolates were found to be defective in inducing GDAR under aerobic growth conditions, while five other isolates were defective in GDAR under aerobic, as well as fermentative, growth conditions. We introduced *rpoS* on a low-copy-number plasmid into 26 isolates and were able to restore GDAR in 20 acid-sensitive isolates under aerobic growth conditions. Four isolates were found to be defective in the newly discovered LuxR-like regulator GadE (formerly YhiE). Defects in other isolates could be due to a mutation(s) in a gene(s) with an as yet undefined role in acid resistance since GadE and/or RpoS could not restore acid resistance. These results show that in addition to mutant alleles of *rpoS*, mutations in *gadE* exist in natural populations of pathogenic *E. coli*. Such mutations most likely alter the infectivity of individual isolates and may play a significant role in determining the infective dose of enterohemorrhagic *E. coli*.

Shiga toxin (Stx)-producing *Escherichia coli* strains are a worldwide cause of human disease, with a wide spectrum of symptoms ranging from mild diarrhea to life-threatening hemolytic-uremic syndrome (HUS). Most of these pathogenic strains possess other virulence characteristics such as the ability to cause attaching-and-effacing lesions on mucosal epithelial cells of the large intestine. The Stx-producing family of human disease-associated *E. coli* is also characterized by its diversity of toxin type (i.e., lysogenic for Stx1-encoding phage or Stx2-encoding phage or with both lysogens) and by its O:H serotype range (21, 29).

In spite of diverse virulence characteristics, one common trait that emerges very clearly is that most of these strains have the ability to withstand gastric acidity (18, 23, 30, 50). In fact, acid tolerance plays a vital role in the survival and virulence of diarrheagenic *E. coli* strains (8, 36, 38). Sigma factor RpoS plays a significant role in survival as it controls several stress-related genes such as catalase, osmotic shock, and heat shock, as well as acid stress (9, 33, 35). Under aerobic growth conditions, *rpoS* regulates two of the three acid resistance pathways (13, 17, 24). However, the ability to survive exposure to acid is a complex phenotype, which depends on the growth phase, medium, and species of enteric bacteria (4, 10, 23). Two types of acid resistance pathways have been identified in the aerobically grown stationary-phase cells of *E. coli* and *Shigella flexneri* (5, 23, 51). The first acid resistance system is referred to as the glucose-repressible oxidative pathway and protects cells above pH 3.0 (23, 24, 38). The structural components of this acid resistance system (other than RpoS), as well as the mechanisms by which it protects the cells, are still unknown (2, 8). The second system is glutamate-dependent acid resistance (GDAR) and can protect cells below pH 3 (19, 38). GDAR requires glutamate decarboxylase (encoded by paralogous genes *gadA* and *gadB*), exogenous glutamate, and an antiporter to release the decarboxylation product γ-aminobutyric acid (encoded by *gadC*) (8). Regulation of the GDAR system is extremely complex. Based on results from multiple gene array studies with *E. coli*, in addition to RpoS and the histone-like protein H-NS, several new regulatory proteins have been implicated to play a role in the expression of *gadA* and *gadBC* and acid resistance (26, 28). For example, GadX and GadW belong to the AraC/XylS-like family of transcriptional regulators and have been shown to affect *gadA*/*BC* expression and acid resistance of cells grown under rich-medium growth conditions (27, 46, 48), whereas GadE (formerly YhiE) is required for acid resistance in cells grown under either rich- or minimal-medium growth conditions (26). In addition, a small RNA molecule as a regulator of acid response genes has also been predicted (34).

Few studies have directly compared the three acid resistance

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† Dedicated to Irving Newman, who passed away on 5 February 2005.

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TABLE 1. Reference *E. coli* strains and plasmids used in this study

Strain or plasmid	Serotype, genotype, original laboratory designation, and other information	Reference or source
52	O157:H7 wild type, ATCC 43895 EK 274; Na^r Rf^r	24
55	Strain 52 *rpoS*::pRR10 Ap^r EK 275	35
65	MG1655 K-12 *rpoS*::Tn*10*, EF 362	8
98	O157:H7 wild type, ATCC 43888	ATCC, Manassas, VA
118	MG1655 K-12 Δ*gadE*::Km, EF1007	26
pACYC184	Low-copy-number cloning vector; Cm^r Tc^r	New England Biolabs, Beverly, MA
pPS4.4	pACYC184 (4.4-kbp ClaI fragment flanking *rpoS*); Cm^r	39
p123	pPCR-Script Amp SK (+) *gadE*	47
pIB1	1.5-kbp BamHI/NotI fragment containing *gadE* from p123 cloned in pACYC184 at BamHI/EagI site; Cm^r	This work
pIB2	4.4-kbp ClaI insert containing *rpoS* from pPS4.4 at ClaI site in pIB1; Cm^r	This work
99–115, 200–265	Pathogenic *E. coli* human isolates except strains 104, 113, 236, 249, and 257 (cow), 259 (pig), 261 (buffalo), and 115 and 260 (meat, hamburger); additional details in Table 3	National Food Safety and Toxicology Center, Michigan State University, East Lansing

systems of pathogenic *E. coli*. For example, 13 of 58 Stx-producing *E. coli* isolates from various sources contained mutated *rpoS* and exhibited increased acid sensitivity; however, acid challenge studies were performed without considering the possibility that exposure to different growth conditions might influence the ultimate outcome of the acid challenge (50). Recent analysis of molecular aspects of acid tolerance pathways in *E. coli* has opened up new strategies capable of dissecting individual acid resistance pathways, as well as identification of additional genes that might influence the acid resistance phenotype. Our aim in this study was to analyze the induction of the GDAR system in various pathogenic *E. coli* strains and investigate the involvement of *rpoS* and other genes in acid tolerance. This has led to a refinement of the acid resistance phenotype in enterohemorrhagic, human-associated, pathogenic *E. coli*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains of *E. coli* used are listed in Table 1. Culture purity of pathogenic *E. coli* isolates was checked by examining the catalase activity (31) of at least 96 colonies of each strain. Frozen stocks maintained at −75°C were streaked on Luria-Bertani (LB) agar, and after overnight growth at 37°C, a single colony was inoculated into minimal E medium (49) containing 0.4% glucose at pH 7.0 (EG minimal medium) (24) or into the complex LB growth medium buffered with 100 mM MES (morpholineethanesulfonic acid, pH 5.5). Most diarrheagenic *E. coli* strains are auxotrophs and require amino acids or vitamins for growth in minimal medium (1, 29). The precise vitamin and amino acid requirements of individual strains were not determined; instead, EG minimal medium was supplemented with 50 μg of yeast extract per ml. Strain 52 is auxotrophic for thiamine, nicotinamide, and riboflavin (1). The strain had the same GDAR phenotype when grown in EG medium supplemented with vitamins or EG medium supplemented with yeast extract (5). Strain MG1655 is not an auxotroph, and the addition of yeast extract to EG medium had no influence on its GDAR phenotype. To obtain stationary-phase cultures, cells were grown in the medium on an orbital shaker (220 rpm, 37°C) for 20 to 22 h to an optical density at 600 nm (OD_{600}) of 3.5 or higher. Strains which were subjected to electroporation with a plasmid (pPS4.4, pIB1, pIB2, or pACYC184) were grown in the medium described above supplemented with chloramphenicol (35 μg ml^{-1}).

For fermentative growth under semiaerobic conditions, cultures were inoculated in brain heart infusion broth with 0.4% glucose (BHIG, 3 ml) in test tubes (13 by 100 mm), which were placed at a 45 ° inclination angle, for 18 to 20 h at 37°C under aeration (shaking at 220 rpm) conditions (10, 23).

Acid challenge and heat shock assays. For the amino acid-dependent acid resistance systems, the cells were diluted directly from the growth medium (1:200) to EG medium (pH 2.0) supplemented with either glutamate (1.5 mM for GDAR or AR2) or arginine (1.5 mM for arginine-dependent acid resistance) and challenged at 37°C for 1 h. EG medium was prewarmed to 37°C, and the pH was adjusted with 6 N HCl (4). Control acid challenge experiments were performed with EG medium (pH 2) without addition of glutamate or arginine. Viable counts were determined after acid challenge by diluting cells in phosphate-buffered saline (50 mM, pH 7.2) and plating immediately on LB agar.

For heat shock assays, stationary-phase cells from LB-MES broth were diluted directly (1:200) into phosphate-buffered saline pre-equilibrated to 58°C. Viable counts were determined at 10-s and 1.5-, 3-, 5-, and 7.5-min intervals by diluting cells in phosphate-buffered saline and plating them immediately on LB agar medium.

Western blot analysis. Cultures were incubated for 18 to 22 h at 37°C to the stationary growth phase (OD_{600} of 3.5 or higher) in EG or LB medium. Cells of each strain were collected by centrifugation (12,500 × *g*, 4 min), washed twice in saline, resuspended at 1 OD_{600} U/ml in 1× loading buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 2.5% β-mercaptoethanol, 0.01% Na-azide, 0.1% bromophenol blue), and placed in a boiling water bath for 5 min to make a whole-cell protein sample. Each protein extract was fractionated on sodium dodecyl sulfate-polyacrylamide gradient gels (4 to 20%). After electrophoretic transfer of proteins onto nitrocellulose membranes, the glutamate decarboxylase isozymes were revealed by using a rabbit primary antibody raised against synthetic polypeptide $_{439}$EDYKASLKYLSDHPKLQ$_{455}$, corresponding to the sequence at the C-terminal end of GadA/B (Research Genetics, Huntsville, AL), which was able to detect both isozymes (52.6 kDa). An anti-rabbit antibody raised in a goat and coupled to peroxidase was used as a secondary antibody. To detect RpoS, nitrocellulose membranes were treated with mouse monoclonal antibody (NeoClone Biotechnology International, Madison, WI) raised against the 38-kDa (RpoS) subunit of *E. coli* RNA polymerase, followed by anti-mouse antibody raised in a goat and coupled to peroxidase as a secondary antibody. A SuperSignal chemiluminescence assay kit (Pierce Biotechnology, Rockford, IL) was used to quantitate the primary antibody, and the signal was captured on preflashed Kodak X-ray film.

Caco-2 cell adherence assays. The human Caco-2 intestinal cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Frozen stock cultures were maintained in GIBCO cell freezing medium held at −140°C. The tissue culture cells were cultivated at 36°C in a 94% air–6%CO_2 atmosphere in minimal essential medium supplemented with Earle's salts, 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. All cell culture media and supplements were obtained from GIBCO (Invitrogen, Gaithersburg, MD).

Adherence assays were performed essentially as described earlier by Kopecko et al. (20). In each well of a 24-well cell culture cluster, approximately 2×10^7 mid-log-phase bacteria (OD_{650} = 0.3 to 0.4) were added to confluent monolayers of about 5×10^5 epithelial cells per well. Adherence was allowed to occur for 1 h at 37°C in a 94% air–6% CO_2 atmosphere. The monolayers were washed three times with Hanks' balanced salt solution and then lysed with 0.1% Triton X-100 in 0.9% saline, and adherent bacteria were enumerated by spread plate count onto Trypticase soy agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD). Adherence ability was expressed as the percentage of the inoculum surviving the washing treatment (i.e., percent recovery). All assays were conducted in quadruplicate and independently repeated at least twice. Results are expressed as an average of the replicate experiments. Recovery percentage data were calculated and analyzed as described previously by using the Student *t* test and one-factor analysis of variance, followed by Dunn or Student-Newman-

TABLE 2. Acid resistance phenotype and basis for classification of strains into four groups

Acid resistance group (no. of strains examined)	% Survival after acid challenge			
	Aerobic growth		Fermentative growth	
			BHIG	
	EG minimal medium, pH 2 + Glu	LB-MES, pH 2 + Glu	pH 2 + Glu	pH 2 + Arg
A (28)	8–53	11–83	21–98	0.4–34
B (20)	0.001–0.2	5–59	19–84	0–41
C (29)	0.001–0.3	0.001–0.4	21–93	0–51
D (5)	<0.001	<0.001	<0.001	2–29

Keuls multiple comparisons of means when significant differences ($P < 0.05$) were found (43).

DNA manipulations and cloning procedures. Standard molecular biology methods were used for chromosomal and plasmid DNA isolation, restriction enzyme digestion and ligation, electroporation, and transformations (37). For cloning of the *gadE* gene from various acid resistance phenotype group D strains, PCR was performed using a 1:9 mixture of Vent and *Taq* DNA polymerase enzymes as previously described (12). PCR products were cloned in pGEMTeasy vector (Promega Corporation, Madison, WI). DNA sequencing was performed using double-stranded plasmid DNA templates using SP6 and T7 primers at the Iowa State University Sequencing Facility (Ames, IA) using a PCR-based dideoxynucleotide terminator protocol and an Applied Biosystems automated sequencer.

RESULTS

Classification of strains based on induction of glutamate-dependent acid resistance. A total of 82 strains were analyzed and represented isolates obtained from 34 countries, including isolates obtained from 23 states within the United States of America. First, all *E. coli* strains were classified into groups based on the induction of GDAR after the strains were grown in either EG minimal medium or LB-MES (pH 5.5) or under fermentative growth conditions (Table 2). Those strains which were able to grow in the EG minimal medium with detectable GDAR levels (survival, ≥1.0%) were classified as acid resistance phenotype group A. There were a total of 28 strains identified in which GDAR was demonstrated to be inducible under these growth conditions (Table 3). Twenty of the 82 strains were able to induce GDAR when grown in LB-MES (pH 5.5) but not when grown in EG minimal medium and were classified as acid resistance phenotype group B (Table 3). None of the 82 strains survived an acid challenge at pH 2 in the absence of glutamate (survival, <0.001%; data not shown). Other strains (29/82) were able to induce GDAR only under fermentative growth conditions (growth on glucose) and were classified as acid resistance phenotype group C (Table 3). Finally, there were five strains which were unable to induce GDAR under any of the induction growth conditions, and these were classified as acid resistance phenotype group D (Table 3). Arginine-dependent acid resistance, which is induced only under fermentative growth conditions, was measured (Table 2) but was not considered for the classification scheme.

As shown in Table 3, there was no correlation between a single acid resistance phenotypic pattern and a dominant lipopolysaccharide-flagellum (O:H) serotype of the diarrheagenic *E. coli* strains. For example, acid resistance phenotype group A consisted of 27 strains with 11 strains representative of well-recognized enterohemorrhagic *E. coli* serotypes, including five O157:H7 strains and one strain each of enterohemorrhagic *E. coli* serotypes O26:HN, O26:H?, O55:H7, and O157:NM and two strains with a serotype designation of O113:H21. Acid resistance phenotype group A also consisted of several strains with traditional enteropathogenic *E. coli* O serogroups or serotypes, including a single O55:H6 strain, five O111 strains with various flagellar antigens, and a single isolate of serotype O119:H6. Acid resistance phenotype group A also contained one enterotoxigenic strain (O128:H7), one enteroaggregative strain (O44:H18), and seven strains with atypical serotype designations, i.e., O103:HN, O104:H21, O111:H9, O121:NM, O128:H21, O128:NM, and O156:H21. Without being too repetitious, the descriptions of the serotype characteristics for the other three acid resistance phenotypic groups are also shown in Table 3.

Glutamate decarboxylase expression profile and glutamate-dependent acid resistance. Next we examined if a correlation existed between the synthesis of glutamate decarboxylase (encoded by *gadA* and *gadB*) and the induction of GDAR across each of the four acid resistance groups (Fig. 1). Both *rpoS*-dependent (aerobic growth to stationary phase in EG and LB-MES media) and *rpoS*-independent (fermentative growth to stationary phase in BHIG medium) induction pathways were examined for three strains from individual acid resistance groups. The glutamate decarboxylase isozymes (GadA and GadB, 52.6 kDa) are 98% identical at the amino acid level (40), and their synthesis was monitored by Western blot analysis using an antibody raised against a synthetic peptide (4, 5).

Strains 210, 237, and 245, which belong to acid resistance phenotype group A, were able to induce GDAR under aerobic growth condition (EG medium, lanes 1 to 3, and LB-MES medium, lanes 4 to 6, respectively) and had correspondingly high level of GadAB proteins (Fig. 1A). The GadAB proteins were not detected in acid resistance phenotype group B strains 205, 212, and 234 grown in EG medium; however, the proteins could be detected from cells grown in LB-MES, as well as in BHIG (Fig. 1B). The cells had functional GDAR, which protected them at pH 2. Strains 200, 218, and 219, which belonged to acid resistance phenotype group C, could synthesize GadAB only under fermentative growth conditions in BHIG medium (panel C) and did not survive an acid challenge of pH 2 if grown in EG or LB-MES medium. The GadAB expression pattern of acid resistance phenotype group C strains matched very closely *rpoS* mutant strains 55 and 65 (Fig. 1, reference strain panel). All five strains belonging to acid resistance phenotype group D were unable to synthesize GadAB in either of the growth media (data not shown).

Influence of functional *rpoS* on GDAR of acid resistance group C strains. The alternative sigma factor (σ^S) encoded by *rpoS* is involved in regulation of the GDAR system in stationary, aerobically grown cells. When grown under fermentative growth conditions, cells induced the GDAR system in an *rpoS*-independent manner. In order to confirm that the defect in GDAR in acid resistance phenotype group C strains is primarily due to erroneous *rpoS*-mediated regulation of GadAB synthesis, we performed genetic complementation tests. By electroporating pPS4.4, which is a moderately low-copy vector construct carrying a functional *rpoS* gene, recombinant strains were tested for GDAR. We were able to electroporate 26 of the 29 acid resistance phenotype group C strains (strains 99, 220, and 232 were resistant to chloramphenicol). Complementation of

TABLE 3. List of foodborne outbreak strains and their classification based on GDAR

AR class	Strain	Serotype	Country or U.S. state	Yr	Original laboratory designation	Note(s)
A	98	O157:H7	Unknown	No data	ATCC43888; CDC B6914-MS1	
A	105	O157:H7	Washington	1993	93-111	1993 Washington outbreak reference strain
A	109	O113:H21	Thailand	1980s	DEC16A	Diarrhea, agglutinin-positive strain BH52C-10
A	110	O104:H21	Montana	1994	G5506	1994 bloody diarrhea outbreak
A	114	O127:H6	United Kingdom	1969	E2348/69	Model EPEC[a] organism
A	207	O26:HN	Switzerland	1952	C240-52	
A	210	O26:H; no data	Chile	1989	VP30	O26:H$^-$ strain from diarrhea
A	211	O44:H18	Peru	1983	042	
A	214	O55:H6	Egypt	1955	F563-55	
A	217	O55:H7	Michigan	No data	3256-97	
A	222	O103:HN	Florida	1997	6:38	
A	224	O111:H2	United Kingdom	1950	DEC12a	DEC12a, diarrhea SSI F1-50
A	225	O111:H2	Peru	1983	DEC12D	
A	228	O111:H9	Finland	1987	921	Diarrhea outbreak
A	235	O111:NM	Panama	1967	DEC12C	
A	236	O111; no data	United Kingdom	No data	C412	Carries invasion insert pLV527
A	237	O113:H21	Canada	1980	CL-3	HUS
A	238	O119:H6	Ohio	1984	277-84	
A	240	O121:NM	Massachusetts	1998	DA-5	
A	242	O128:H7	Tanzania	1974	DEC13C	
A	245	O128:H21	Bangladesh	1977	DEC14E	
A	246	O128:NM	Virginia	1979	DEC14D	
A	249	O156:H21	Germany	No data	M2113	Bovine isolate, *stx1*$^+$
A	251	O157:H7	Washington	1993	93-111	1993 Washington outbreak
A	257	O157:H7	Argentina	1977	DEC4A	
A	258	O157:H7	Denmark	1987	DEC4B	
A	259	O157:H7	Egypt	1983	DEC4C	
A	261	O157:NM	California	1986	DEC7E	
B	101	O11:NM	Nebraska	1985	3007-85	O111:H$^-$ strain from HUS
B	102	O26:H11	Delaware	1977	DEC10C	O26:H11 strain from infant
B	103	O26:HNM	South Dakota	1974	DEC9F	Nontoxigenic O26:H$^-$
B	106	O157:H7	California	1975	2886-75	First U.S. case of disease
B	108	O55H7	Washington	1991	TB182A	Nontoxigenic O55:H7 relative of O157:H7
B	111	O91:H7	United Kingdom	1967	23/67	ID[b] outbreak, EPEC strain 23/67
B	112	O103:H6	Washington	1991	TB154A	Diarrhea, fecal leukocytes
B	113	O5:HN	California	No data	BCL17	Bovine isolate, diarrhea
B	115	O55:H7	No data	1994	5905	*stx2*$^+$ O55:H7
B	201	O26:H11	Mexico	1986	45	
B	205	O26:H11	Delaware	1977	DEC10C	
B	208	O26:HN	New Hampshire	1979	DEC9B	
B	212	O55:H6	Pennsylvania	1956	DEC1a	ID outbreak, CDC 572-56
B	229	O111:H11	Cuba	1953	DEC8D	
B	231	O111:H12	Guatemala	1967	DEC6C	
B	234	O111:HN	Kenya	1986	DEC12E	
B	250	O157:H7	Japan	1996	OK-1	1996 Okayama, Japan outbreak
B	254	O157:H7	New Mexico	1988	DEC3C	
B	260	O157:H7	Oregon	1982	EDL-933	
B	262	O167:H?	Brazil	1981	LT-82	
C	99	O11:H8	Canada	1982	CL-37	O111:H8 strain from HUS
C	100	O11:H8	Idaho	1986	DEC8b	O111:H8 strain from HC[c]
C	104	ON:NM	California	No data	BCL19	Feedlot calf died of diarrheal disease
C	107	O157:H	Germany	1989	493/89	Sorbitol fermenting, nonmotile O157:H$^-$
C	200	O26:H11	Australia	1986	DEC10B	O26:H11 strain from HC
C	202	O26:H11	United Kingdom	No data	H30	
C	203	O26:H11	France	1952	C12-52	
C	204	O26:H11	Wisconsin	1961	DEC9A	
C	213	O55:H6	Dutch Guiana	1958	DEC1B	
C	215	O55:H6	Congo	1962	DEC2a	
C	216	O55:H7	Sri Lanka	1965	DEC5D	Nontoxigenic O55:H7 relative of O157:H7
C	218	O55:H7	Sri Lanka	1965	DEC5D	Diarrhea, C586-65
C	219	O55:H7	New York	1950	DEC5A	
C	220	O55:H7	Iran	1963	DEC5E	
C	221	O91:H21	Canada	1985	B2F1	HUS, mucus-activated Stx
C	226	O111:H8	Idaho	1986	DEC8b	HC *rpoS* lesion, CDC 3030A-86

Continued on following page

TABLE 3—*Continued*

AR class	Strain	Serotype	Country or U.S. state	Yr	Original laboratory designation	Note(s)
C	230	O111:H12	Germany	1954	C142-54	
C	232	O111:H12	Italy	1951	F436-51	
C	233	O111:H21	New Jersey	1966	DEC6A	
C	239	O121:H19	Montana	1998	MT#2	
C	244	O128:H21	India	1971	DEC14B	
C	247	O142:H6	Brazil	1983	1181-83	
C	252	O157:H7	Japan	1996	OK-1	1996 Okayama, Japan, outbreak
C	253	O157:H7	Washington	1985	DEC3A	
C	255	O157:H7	Michigan	1988	DEC3D	
C	256	O157:H7	Canada	1988	DEC3E	
C	263	O128a:H2	Missouri	1971	DEC11B	
C	264	No data	Maryland	1967	3408-67	
C	265	No data	Vietnam	1953	3615-53	
D	206	O26:H?	Washington	1991	TB285C	O26:H$^-$ strain from diarrhea
D	209	O26:HNM	Rhode Island	1994	395-2	
D	227	O111:H8	Texas	1999	3215-99	
D	241	O125:HNM	North Carolina	1986	3288-85	HC
D	248	O145:HNM	South Dakota	1982	75-83	

[a] EPEC, enteropathogenic *E. coli*.
[b] ID, infantile diarrhea.
[c] HC, hemorrhagic colitis.

functional *rpoS* by electroporation restored GDAR under aerobic growth conditions in most of the acid resistance phenotype group C strains, except for strains 100, 104, 226, 244, 252, and 255 (data not shown). From this subgroup, strains 226 and 255 were selected for further analysis (see below).

In comparison to their wild-type parental strains, recombinant strains 107, 230, 218, and 219 carrying pPS4.4 synthesized RpoS, which could be detected by Western blot analysis (Fig. 2A). (The wild-type and corresponding recombinant strains carrying pPS4.4 are referred without and with a -1 suffix, e.g., strains 107 and 107-1, respectively.) Concomitantly, the strains synthesized glutamate decarboxylase and were able to induce GDAR when grown aerobically in LB-MES. In the majority of the acid resistance phenotype group C strains, synthesis of RpoS could not be detected by Western blot analysis. On the other hand, RpoS from strains 226, 247, 255, and 256 could be detected by Western blot analysis (Fig. 2B). The translation levels of the protein were clearly reduced in strains 255 and 256 (Fig. 2B, lanes 1 and 3) but were significantly higher for the other two strains (lanes 5 and 7). In spite of their RpoS levels, the strains were unable to synthesize the GadAB proteins and could not induce GDAR when grown to stationary phase in LB-MES medium. Upon electroporation with pPS4.4, much higher levels of RpoS could be detected in strains 255-1 and 256-1 (Fig. 2B, lanes 1 and 3 versus 2 and 4, respectively), while there was no discernible difference in the RpoS levels of strains 247-1 and 226-1 (Fig. 2B, lanes 5 and 7 versus 6 and 8, respectively). However, only strains 256-1 and 247-1 were able to synthesize GadAB, as well as induce GDAR (Fig. 2B, lanes 2 and 6), while other recombinant strains (226-1 and 255-1) remained defective in GDAR during aerobic growth (Fig. 2B, lanes 4 and 8).

In order to ascertain that pPS4.4 encoded catalytically active RpoS in strains 226 and 255, we compared the temperature tolerances of the wild-type and recombinant strains at 58°C (Fig. 3). The survival patterns of strains 255 and 256 were very similar, and both strains acquired considerable temperature tolerance upon receiving pPS4.4, a phenotype indicative of successful *rpoS* expression in both strains. Similarly, heat shock analysis of strains 247 and 226 indicated that strain 247-1 gained heat tolerance along with GDAR, while strain 226 was heat tolerant to begin with and remained so after receiving pPS4.4.

Effect of functional *gadE* on induction of GDAR of acid resistance phenotype group D strains. The inability of the acid resistance phenotype group D strains to induce GDAR even under fermentative growth conditions indicated that the cells were not able to utilize either of the *rpoS*-independent or *rpoS*-dependent pathways. The recently discovered LuxR-like regulator GadE (formerly YhiE) is suggested to be required in addition to the *rpoS*$^+$ genetic background for the aerobic induction of GDAR (26). We analyzed the DNA sequence of the *gadE* gene from strains 206, 209, 227, 241, and 248 and examined functional complementation by mobilizing plasmid pIB1 (*gadE*) by electroporation (Table 4). All acid resistance phenotype group D strains, with the exception of 209, had a defect in their *gadE* sequence; however, only two strains were complemented for GDAR when the *gadE* gene was mobilized by electroporation. The complementation in strain 248 was fully successful, as *gadE* restored the aerobic (*rpoS*-dependent) and fermentative (*rpoS*-independent) induction pathways. The ability of strain 227 (pIB1) to induce GDAR only under fermentative growth conditions indicated the lack of functional *rpoS*, and to confirm this possibility, pIB2 (*rpoS gadE*) was mobilized to strain 227. With both regulators being functional, strain 227(pIB2) was fully restored in GDAR induction (Table 4). Two other strains, 206 and 241, remained defective for GDAR in spite of complementing the defective *gadE* and/or *rpoS* gene(s).

Influence of *gadE* and *rpoS* on Caco-2 cell adherence properties of acid resistance phenotype group D strains 227 and 248. Recently, Tatsuno et al. (44) reported increased adherence to Caco-2 cells due to disruption of the *yhiE* (now *gadE*) and *yhiF* genes in enterohemorrhagic *E. coli* O157:H7. We

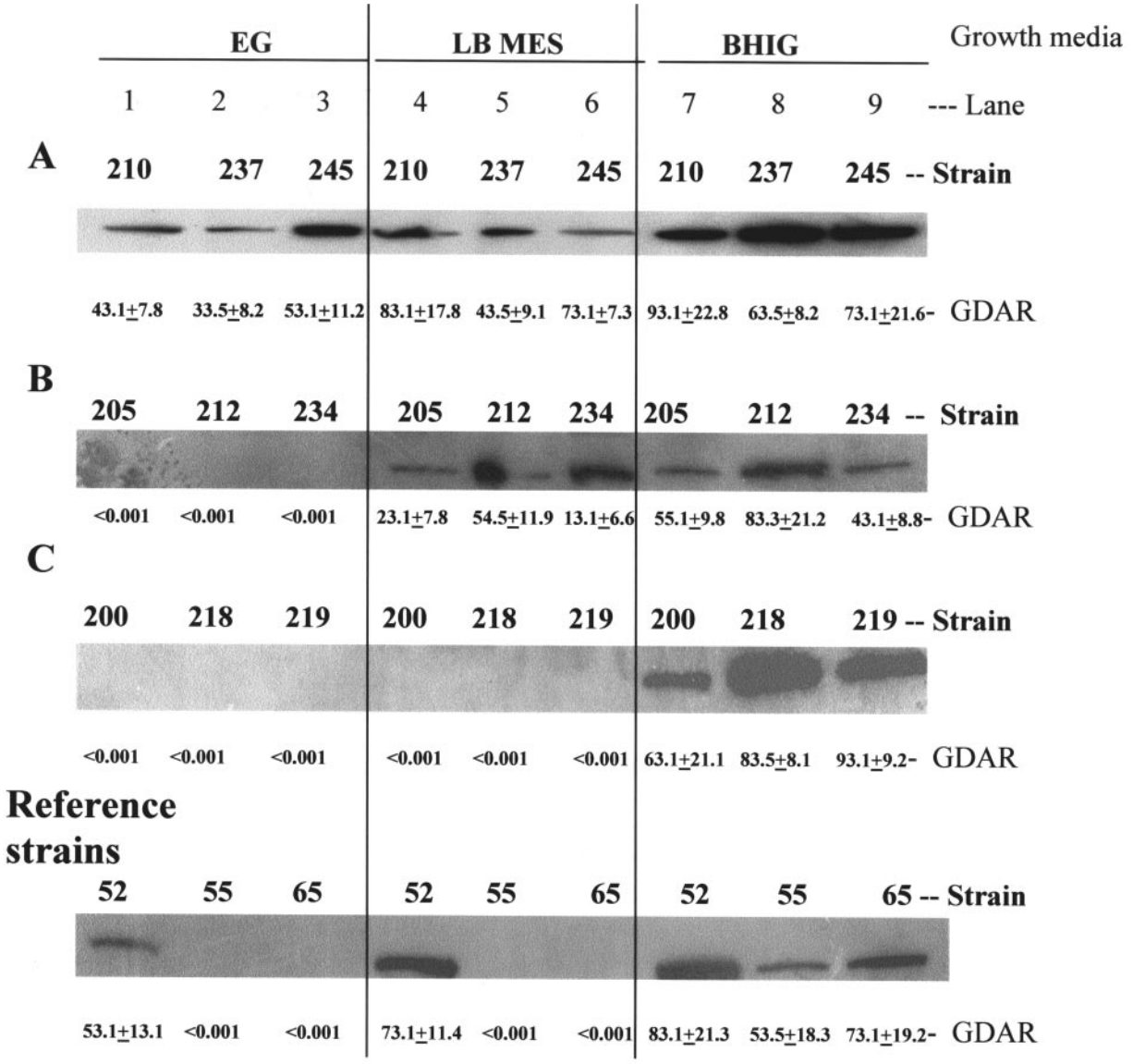

FIG. 1. Effect of growth conditions on the synthesis of glutamate decarboxylase isozymes (GadAB) and expression of GDAR in various *E. coli* strains. *E. coli* strains were grown to the stationary phase in EG medium (lanes 1 to 3), LB-MES medium (lanes 4 to 6), and BHIG medium (lanes 7 to 9), and equivalent amounts of protein were loaded into each lane and probed with the anti-Gad antibody to detect GadAB (52.6 kDa). The cells were also subjected to an acid challenge in the presence of 1.5 mM glutamate at pH 2 for 1 h, and the percentage of the population surviving is indicated below each lane (with the standard deviation of the mean). Individual strains used are indicated in bold above each lane. Panels: A, acid resistance phenotype group A strains; B, acid resistance phenotype group B strains; C, acid resistance phenotype group C strains. The data for the reference strains are shown in the bottom panel.

examined strains 227 and 248, which carried a mutation in the *gadE* gene, for their adherence properties on Caco-2 cells (Fig. 4). *Salmonella enterica* serovar Typhi strain Ty-2 was used as a positive control, and *E. coli* HB101 adhesion was considered a baseline reading. Contrary to the findings of Tatsuno et al. (44), strain 248 was not more adherent to Caco-2 cells; in fact, strain 248(pIB1) adhered slightly better than strain 248. Similarly, strain 227 and strain 227(pIB1) both adhered to Caco-2 cells at reduced efficiency and were not significantly different than strain HB101. Since strain 227 had mutations in *gadE* as well as *rpoS*, we examined 227(pIB2) and 227(pPS4.4) for cell adhesion in order to gauge the influence of individual regulators. Collectively, both regulators positively influenced adhesion of strain 227; however, the influence of *gadE* was observed only in the *rpoS*$^+$ genetic background. The other two acid resistance phenotype group D isolates which carried a mutation in *gadE*, strains 206 and 241, adhered at 0.47% ± 0.26% and 1.44% ± 0.76% efficiency. Strain 209, with the wild-type *gadE* sequence, adhered at 3.09% ± 1.37% efficiency.

DISCUSSION

In the most comprehensive single study, over 20% (13/58) of Stx-producing *E. coli* isolates were reported to carry a muta-

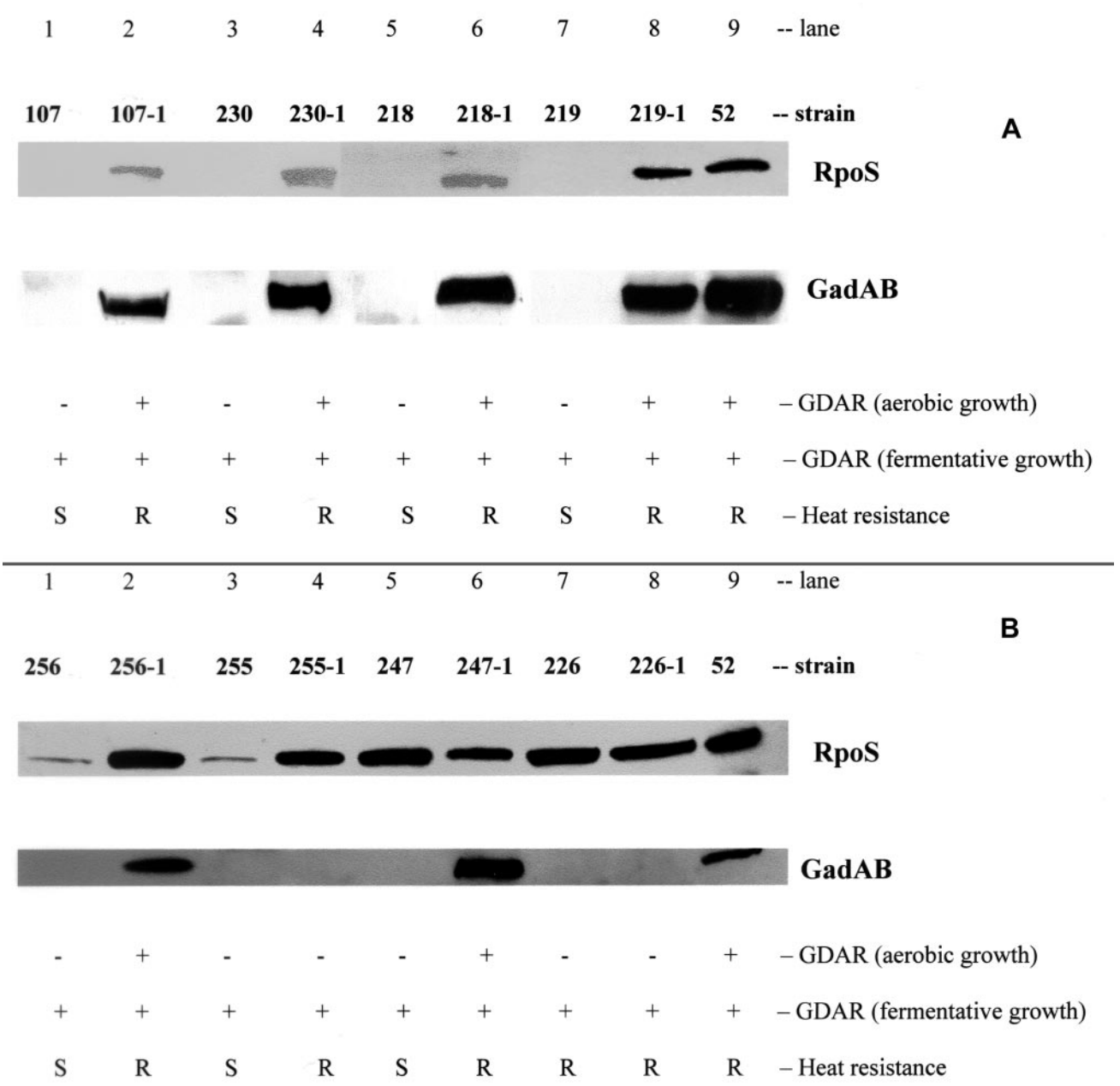

FIG. 2. Western blot analysis of GadAB versus RpoS expression in acid resistance phenotype group C strains. Wild-type *E. coli* strains and corresponding recombinants carrying pPS4.4 (*rpoS*) were aerobically grown to the stationary phase in LB-MES medium, and equivalent amounts of protein were loaded into each lane and probed with the anti-RpoS and anti-Gad antibody to detect RpoS (38 kDa) and GadAB (52.6 kDa). RpoS and GadAB expression from cells was measured after aerobic growth to stationary phase in LB-MES medium. GDAR was measured after growing cells in aerobic (LB-MES) and fermentative (BHIG) growth media; a plus sign indicates that the surviving population is >1.0%, and a minus sign indicates that the surviving population is <1.0%. The heat resistance (58°C for 7.5 min) phenotype was determined after growing cells aerobically in LB-MES growth medium, and the data (from Fig. 3) are indicated as R (surviving population, >1.0%) or S (surviving population, <1.0%). (A) Strains 107, 230, 218, and 219 (lanes 1, 3, 5, and 7, respectively); their recombinant derivatives carrying pPS4.4 (*rpoS*), i.e., 107-1, 230-1, 218-1, and 219-1 (lanes 2, 4, 6, and 8, respectively); and reference strain 52 (lane 9). (B) Strains 256, 255, 247, and 226 (lanes 1, 3, 5, and 7, respectively); their recombinant derivatives carrying pPS4.4 (*rpoS*), i.e., 256-1, 255-1, 247-1, and 226-1 (lanes 2, 4, 6, and 8, respectively); and reference strain 52 (lane 9).

tion in *rpoS* and had reduced acid tolerance (50). In yet another study, a wide range of acid tolerances among different strains of *E. coli* O157:H7 and *E. coli* non-O157:H7 isolates was recorded (7). Although studies like these have contributed substantially to our knowledge of acid tolerance of pathogenic *E. coli*, a more detailed analysis aimed at dissecting individual acid resistance pathways is needed. Since all available evidence suggests that induction of acid resistance occurs prior to ingestion, we analyzed 82 pathogenic *E. coli* strains for the ability to induce acid resistance systems under different physiological

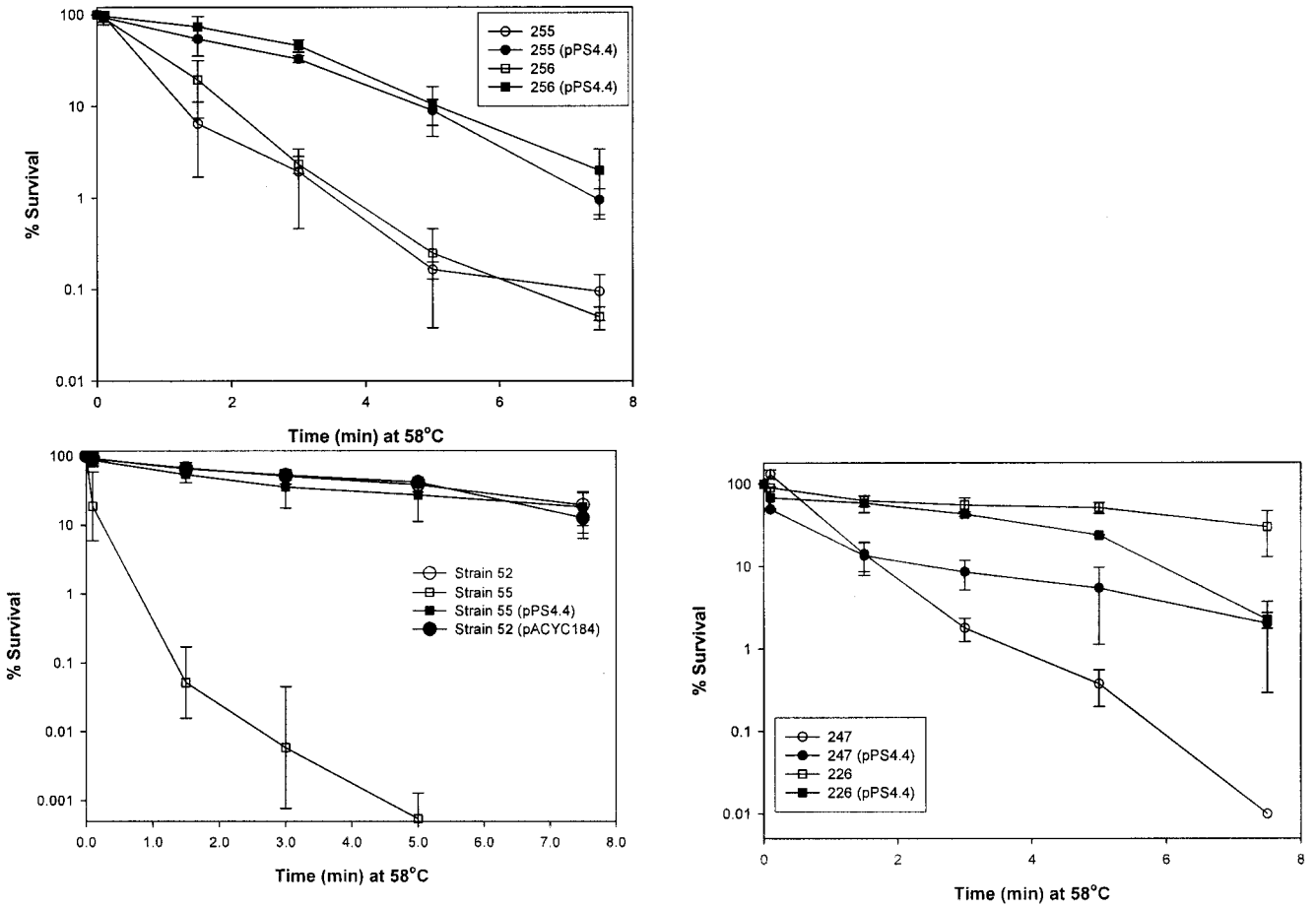

FIG. 3. Effect of *rpoS* mutations on heat tolerance of acid resistance phenotype group C strains. *E. coli* strains were grown to the stationary growth phase in LB-MES medium and diluted 1:200 in phosphate-buffered saline pre-equilibrated at 58°C. The surviving population was determined at the indicated time intervals by withdrawing samples and plating them immediately on LB agar (for wild type strains) or LB agar containing chloramphenicol (35 μg ml^{-1}) for recombinant derivatives carrying pPS4.4 (*rpoS*).

conditions and particularly analyzed and compared induction of GDAR among different strains.

In the beginning, we classified strains on the basis of the ability to induce GDAR when grown in EG minimal medium. Comparative genome sequence analysis studies have indicated that most pathogenic *E. coli* strains lack up to 10% of the backbone DNA present in K-12 strains (14). We found that approximately 60% of the strains (acid resistance phenotype group B and group C; 49/82) had an auxotrophic requirement for GDAR induction in EG medium that could not be met with 50 μg ml^{-1} yeast extract and could induce this acid resistance system only in complex medium such as LB-MES or BHIG. Most strains (94%; 77/82) were able to induce GDAR under either aerobic or fermentative growth conditions (all strains except those in acid resistance phenotype group D). Both regulatory pathways, RpoS dependent and RpoS independent for GDAR induction, were defective in acid resistance phenotype group D strains (6% of strains, 5/82). However, the arginine-dependent acid resistance pathway was operative in all acid resistance phenotype group D strains. Thus, the availability of multiple acid resistance pathways ensures the pathogen's life style and at the same time might explain the discrepancies observed in acid resistance and/or the infective dose among various strains as different acid resistance systems may be required to be operative under commensal or pathogenic lifestyles (35, 36).

We found that 29 out of 82 isolates were defective in the aerobic induction of GDAR, which requires functional *rpoS*. The general stress resistance of *E. coli* is controlled by the RpoS sigma factor, but mutations in *rpoS* are surprisingly common in natural and laboratory strains (11, 22, 25, 50). The questions of whether there is any possibility of a selective advantage in losing *rpoS* and whether RpoS contributes to fitness under nutrient limitations are being actively investigated by several laboratories (15, 16, 22, 32). We analyzed the strains with a putative defect in *rpoS* by a physiological and recombinant DNA approach. Out of the 26 acid resistance phenotype group C strains we electroporated with pPS4.4 (*rpoS*), 20 strains restored their ability to synthesize RpoS and GadAB, as well as the ability to induce GDAR under aerobic growth conditions. However, due to constrains of the Western blot assay technique, only limited information could be drawn from strains 256, 255, 247, and 226, which exhibited various levels of RpoS expression, and their GDAR expression was not

TABLE 4. Effect of functional *gadE* and *rpoS* on GDAR of acid resistance phenotype group D strains

Strain and change in *gadE* nucleotide sequence	Change in amino acid sequence	% Survival (mean ± SD) after acid challenge of cells grown under the indicated condition	
		Aerobic	Fermentative
MG1655 (wild type)	175 aa[a]	33 ± 8.2	45.1 ± 11.1
118 (*gadE*::Km)	Knockout	<0.001	<0.001
118(pIB1)		27 ± 7.5	31.6 ± 6.2
206: T deleted at 9	Truncation; retains first 2 aa + 2 nonsense aa	<0.001	<0.001
206(pIB1)		<0.001	<0.001
206(pIB2)		<0.001	<0.001
227: C insertion at 46	Truncation; retains first 15 aa + 5 nonsense aa	<0.001	<0.001
227(pIB1)		<0.001	31.3 ± 4.5
227(pIB2)		21.9 ± 6.5	43.1 ± 7.3
248: A deleted at 105	Truncation; retains first 35 aa + 1 nonsense aa	<0.001	<0.001
248(pIB1)		24.1 ± 7.1	33.2 ± 4.4
209: no defect	175-aa wild-type protein	<0.001	<0.001
209(pIB1)		<0.001	<0.001
209(pIB2)		<0.001	<0.001
241: C inserted at 8	Truncated, retains first 2 aa + 18 nonsense aa	<0.001	<0.001
241(pIB1)		<0.001	<0.001
241(pIB2)		<0.001	<0.001

[a] aa, amino acids.

uniform once they acquired functional *rpoS*. The reason for the lack of GDAR expression in strains 247 and 226 does not appear to be weak translation or suboptimal quantities of RpoS (Fig. 2B, lanes 5 and 7, respectively), although the possibility remains that the RpoS protein is dysfunctional.

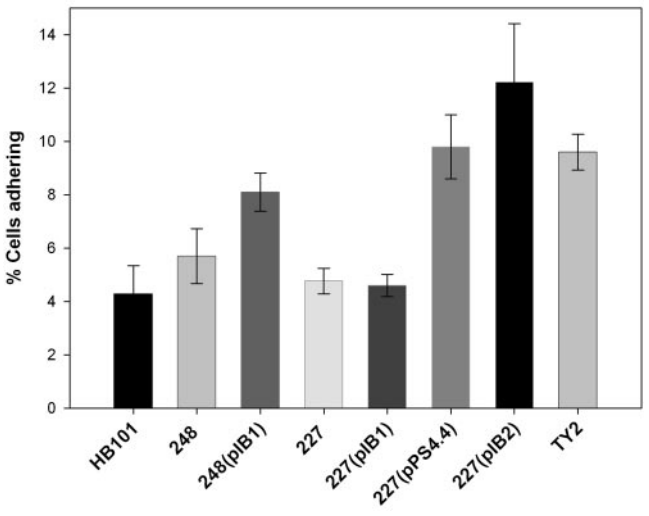

FIG. 4. Adherence phenotype of acid resistance phenotype group D strains 227 and 248. Bacteria grown to mid-log phase were used to infect Caco-2 cell monolayers and were allowed to adhere for 1 h at 37°C in a 94% air–6% CO_2 atmosphere. The monolayers were washed three times with Hanks ' balanced salt solution and then lysed with 0.1% Triton X-100 in saline. The adherent bacteria (enumerated by spread plate count) are expressed as the percentage of the inoculum surviving washing treatment (percent recovery). All assays were conducted in quadruplicate and independently repeated at least twice. Error bars indicate the standard deviation of the mean.

In order to overcome this limitation of the Western blotting technique, we took advantage of the fact that *rpoS* also regulates heat tolerance and used the heat tolerance assay to gauge the functionality of native and recombinant RpoS (Fig. 3). In spite of detectable levels of RpoS, strain 247 belonged to acid resistance phenotype group C and was heat sensitive. However, the strain gained GDAR, as well as heat tolerance, upon electroporation with pPS4.4, indicating that RpoS from strain 247 could be catalytically inactive. On the contrary, strain 226 does not appear to be defective in *rpoS* based on its heat tolerance ability (Fig. 3) and it is likely that strain 226(pPS4.4) could be overexpressing RpoS, creating an imbalance in the cell's heat tolerance. It is likely that wild-type strain 226 could be defective in GDAR due to a lack of a regulatory element(s) yet to be identified for aerobic induction. Further more, strain 255 also appears to carry a defect(s) in another regulatory gene(s) in addition to *rpoS* since in spite of successful expression of RpoS from pPS4.4 (i.e., complementation for heat tolerance), this strain remained defective in GDAR (Fig. 2B). Recently, a small regulatory RNA (*gadY*) was identified in *E. coli* K-12 (MG 1665) whose expression is dependent on *rpoS* and which is predicted to have a role in the enhanced expression of *gadX* and downstream acid resistance genes (34). Strains 226 and 255 need to be examined for any mutations in *gadY*; alternatively, the strains could be used as trap hosts to identify regulatory elements for aerobic induction of the GDAR system. It may be noted that all acid resistance phenotype group C isolates were able to induce GDAR by an *rpoS*-independent pathway when they were grown under fermentative conditions, indicating a fully functional GDAR system (Table 2).

Four isolates from acid resistance phenotype group D were defective in a newly discovered LuxR-type positive regulator,

gadE. However, mobilization of pIB1 (*gadE*) could restore GDAR in strain 248 while in strain 227 it required fermentative growth, indicating a possible defect in *rpoS* (Table 4), which was later confirmed by complementation using pIB2 (*rpoS gadE*). The reasons for the inability to induce GDAR (under either aerobic or fermentative growth conditions) by three of the group D isolates (strains 206, 209, and 241) are not apparent at this time. All group D isolates were examined for the presence of *gadBC* and *gadXW* regions by PCR amplification (data not shown). Although we could detect identical-size PCR amplification products on agarose gels with reference to the wild-type strains, the possibility of a point mutation(s) or smaller deletions cannot be ruled out. Alternatively, the strains could be lacking regulators yet to be identified for the *rpoS*-independent induction of GDAR. The possible involvement of *yhiE* (*gadE*) in negatively regulating adhesion to intestinal cells in *E. coli* O157:H7 (44) prompted us to examine group D strains in Caco-2 cell assays. However, we were unable to detect a negative regulatory role of *gadE* in adhesion to Caco-2 cells. In fact, we observed a synergistic effect of two regulators, and strain 227(pIB2) was most efficient in adhering to Caco-2 cells. Strains 227 and 248 belonged to serogroups O111:H8 and O145:HNM, respectively, and in addition to the involvement of multiple elements in controlling the adherence of *E. coli* O157:H7 (45), it appears that regulation of adhesion mechanisms differs among O157 and other serogroups (42).

The *rpoS* mutations are much more prevalent in natural populations, and certainly *mutS* and *rpoS* are located in a highly polymorphic segment of the chromosome (6, 11, 16). In studies in which pathogenic or commensal *E. coli* isolates were analyzed, several sequence variants of *rpoS* were reported and a range of phenotypes attributable to altered *rpoS* function were exhibited (3, 9, 22). Based on batch and chemostat culture studies, it has been proposed that mutant *rpoS* alleles can confer a survival advantage over wild-type strains (16, 22). The GDAR system provides the highest level of protection, functioning at pH 2 or less, and can be induced in an *rpoS*-independent manner (2, 8). GDAR may also be very important for food safety, as monosodium glutamate will support acid resistance although it is intended to be a food preservative. In order to extend shelf life, more and more ready-to-eat food is marketed in modified-atmosphere packages (41, 52). It is necessary to determine if such an environment would provide fermentative conditions for induction of GDAR in an *rpoS*-independent manner.

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